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Effect of Ototoxic Drugs on the Amphibian Auditory System: Injection of Gentamicin Sulfate into Anuran Otic Capsules and Recovery of Thresholds

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THESIS APPROVAL

The abstract and thesis of Michael Patrick Brown for the Master of Science in Biology were presented on May 17, 1995 and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Michael Patrick Brown for the Master of Science in Biology presented May 17, 1995.

Title: Effect of Ototoxic Drugs on the Amphibian Auditory
System: Injection of Gentamicin Sulfate into
Anuran Otic Capsules and Recovery of Thresholds

Hair cell trauma from aminoglycosides, which may lead to permanent loss of hair cells in mammals, was studied physiologically in frogs by measuring an auditory evoked potential (AEP) in *Rana pipiens*. The AEP was evaluated in order to measure threshold shift (TS) and recovery from TS after the administration of the aminoglycoside antibiotic, gentamicin. To obtain an AEP, chronic electrodes were implanted into the cranium near the cochlear nucleus. The frogs were then exposed to frequency-specific narrow band "clicks" which included a single period 1 kHz sinewave, and a computer synthesized high frequency and low frequency click. Amphibians have two hearing organs, the amphibian and basilar papillae, sensitive to low (150-1500) and high (1500-2000) frequencies, respectively. The low (AP) and

high (BP) frequency clicks were created to stimulate specifically the corresponding papillae. After normal thresholds were recorded for each frog, gentamicin sulfate, 200 μM , 300 μM , or 400 μM , was injected bilaterally into the otic capsules. Thresholds were recorded until the TS had disappeared, allowing the threshold recovery period to be measured. The injections of 200 μM yielded a 10 dB change in one animal and no change in two others. The injection of 300 μM into 10 frogs and 400 μM into 20 frogs yielded at least a 10 dB change in 60% and 93% of the frogs, respectively, with the concentration of 400 μM producing threshold shifts of 20 dB. Thus, the threshold shifts were dose-dependent. Recovery times varied between six and fourteen days. No apparent differences between thresholds for the high frequency click, low frequency click and sinewave clicks were observed.

**EFFECT OF OTOTOXIC DRUGS ON THE AMPHIBIAN AUDITORY SYSTEM:
INJECTION OF GENTAMICIN SULFATE INTO ANURAN
OTIC CAPSULES AND RECOVERY OF THRESHOLDS**

by

MICHAEL PATRICK BROWN

A thesis submitted in partial fulfillment of the
requirements for the degree of

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in

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Introduction

In our quest to relieve the pain and suffering of people through the use of drugs, even though the objective may be reached, unexpected repercussions may occur. It has long been known that treatment with one class of antibiotic, the aminoglycosides, can cause damage to the auditory system (Girod and Rubel, 1991). It has been estimated that in the United States hearing impairment, frequently irreversible, occurs in as many as 5% of patients who have received aminoglycosidic treatment (Kroese et al., 1989). Despite this negative consequence, the particular effectiveness of aminoglycosides supports their continued use. For example, aminoglycoside antibiotics are used primarily in the treatment of meningitis and other serious infections (Arritola, 1995; Duff, 1992).

Hair cells of the inner ear transduce motion into neural signals for further nervous system processing and are the elements apparently damaged following chemical or acoustic trauma (Lefebvre et al., 1993; Lippe et al., 1991; Pugliano et al., 1993a). In most studies, the

amount of hair cell damage is assessed by sacrificing the animal at specific periods post-treatment and, utilizing electron microscopy, viewing hair cells directly (Baird et al., 1993; Forge et al., 1993; Raphael, 1993; Cotanche and Lee, 1994). This method provides a direct correlation between the amount of trauma induced and the number of hair cells damaged. A few studies have examined physiological measures of damage, but no studies in amphibians have measured physiological changes in response to ototoxic drugs (Baird et al., 1993; Kroese et al., 1989).

In this study, I examine the effects of one aminoglycoside antibiotic, gentamicin, on the hearing of the anuran amphibian, *Rana pipiens*. Unlike several other studies, rather than systemic injection, the aminoglycoside was injected bilaterally into the otic capsules (Baird et al, 1993; Hashino et al., 1992; Lombarte et al., 1993). The otic capsule is a bony region of the head containing the auditory papillae. The drug's effect was monitored utilizing an auditory brainstem recording (ABR) method in which electrodes were implanted near the brainstem to detect volume-conducted potentials from the VIII cranial nerve and ascending auditory nerve

nuclei. Because the electrodes were implanted chronically, this method allows measurement of frog hearing threshold, in most cases, up to five weeks. Depending on the concentration, the treatments resulted in temporary threshold shifts (TTS), with a return to normal threshold within 7-14 days.

Frogs possess two hearing organs in each ear, the amphibian papilla (AP) and basilar papilla (BP) (Lewis et al., 1992). Even though there are actually eight sensory organs with hair cells, only the AP and BP respond to acoustic stimuli. The other six are vestibular organs and include three semicircular canals, utricle, saccule, and lagena (Wilczynski and Capranica, 1984). The AP is unique to amphibians and is generally larger than the BP. The AP is most sensitive to low frequencies and the BP to higher frequencies (reviewed in Wilczynski and Capranica, 1984). Because the AP and BP are two separate organs, it is possible that aminoglycosides affect each organ differently. For example, the high frequencies may be affected more than the low frequencies. This is the first study to attempt to stimulate the AP and BP separately using an ABR technique. This was accomplished using

computer generated high frequency and low frequency
clicks.

Literature Review

Hair Cells:

Vertebrates have developed efficient neural sensory systems for the processing of sound. One feature common to these systems is the hair cell. When appropriately stimulated, this mechanoreceptive sensory cell converts changes in sound pressure to electrical signals. These neural signals are then transmitted to the higher centers of the brain for processing. In mammals, the hair cells are arranged on the cochlea such that they are "tuned" to specific frequencies. This phenomenon is referred to as tonotopic organization and is preserved in the neural pathway to the brain. However, tonotopy is not present in all auditory organs of different species (Cotanche et al., 1994; Corwin and Warchol, 1991).

Somewhat independent of tonotopic organization is the orientation of the hair cells. Each hair cell body has a base on which there is a bundle of stereovilli. The stereovilli determine the structural and functional characteristics of the bundle (Corwin and Warchol, 1991). In mammals, there exist four highly ordered rows of hair cells in the cochlea. However, not all animals have a

cochlea with this arrangement. For example, birds, reptiles and amphibians lack cochleas (Corwin and Warchol, 1991; Corwin et al, 1993). Instead, a simpler papilla is found in place of the more complex cochlear organ of Corti and basilar membrane. Hair cells often vary in their orientation on a papilla and may appear to be randomly dispersed. In fish, hair cells are arranged in various ordered configurations in the sacculus (Lombarte et al., 1993; Presson et al., 1995; Weisleder and Zakon, 1995). Birds possess a papilla with ordered, tuned hair cells which are sensitive to specific frequencies (Corwin and Warchol, 1991).

In most frog species, the hair cells of the AP are distributed along a long "S"-shaped organ. The hair cells themselves possess a bundle of stereovilli with a longer kinocilium at one end of the bundle. The AP has an ordered arrangement of hair cells (Schmitz et al., 1992). However, the specific orientation of the hair cells may vary along different regions of the papilla (Lewis et al, 1992). The hair cells of the BP are arranged in a semicircle, and the kinocilium on each hair cell, is located on the side of the bundle with the shortest stereovilli. The BP hair cells all have an identical

orientation (Wilczynski and Capranica, 1983). The AP supports a traveling wave and neural tonotopy. However, tonotopy is not a characteristic of the BP (Corwin and Warchol, 1991; Hillery and Narins, 1984).

Hair cells may be damaged by two mechanisms, acoustic overstimulation and ototoxicity, as caused by drug treatments (Abdouh et al., 1993; Aran et al., 1995; Duckert and Rubel, 1993a). Acoustic overstimulation caused by noise exposure is a universal concern today because of exposure to loud music and machinery noise. However, ototoxicity caused by drug treatments is fast becoming a major problem faced by the medical community. The loss of hair cells by this means is responsible for hearing and balance deficits that affect millions of people (Mandell et al., 1990). Problems associated with vestibular toxicity include vertigo, disequilibrium, lightheadedness, nausea, and vomiting. Ototoxicity can lead to tinnitus (ringing) and temporary or permanent hearing loss (Smith and Swenson, 1990; Tsue et al., 1994). It has been estimated that approximately 5% of the population has hearing impairment as the result of the destruction of hair cells (Kroese and Bercken, 1982).

Aminoglycosides:

Many drugs sold today may cause ototoxicity as a side effect (Duckert and Rubel, 1993a). Aminoglycosides fall into this category. Unfortunately, sometimes the need for the medication outweighs the risk of possible side effects. Aminoglycoside antibiotics are the drugs of choice for bacterial meningitis and many other serious infections (Duff, 1992; Ernst and Zenner, 1992). Their main target is aerobic gram-negative bacilli, namely *Pseudomonas* species and virtually all strains of *Streptococcus faecalis* (Mandell et al, 1990). Besides nephrotoxicity and neuromuscular paralysis, all aminoglycosides are capable of causing ototoxicity which can lead to permanent hearing loss. There are several types of aminoglycosides which may affect hair cell organs differently. The mammalian vestibular system is primarily susceptible to damage by streptomycin and gentamicin, while amikacin, kanamycin, and neomycin primarily affect the cochlear system (Mandell et al., 1990; Wanke and Moore, 1991). In fact, approximately 25% of the patients under streptomycin treatment experience vertigo or other vestibular disturbances (Smith and Swenson, 1990).

On the molecular level, aminoglycoside antibiotics contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol ring. The primary difference between the aminoglycosides is in the nature of the hydroxyl and aminosugar groups that are linked to the central aminocyclitol ring (Mandell et al., 1990). In general, the mechanism of action is the inhibition of protein synthesis by binding directly to the 30S ribosomal subunit (Arritola, 1995). This kills the bacteria. The exact mechanism of action on the sensory hair cells is still under investigation, but it appears that the sensory hair cell membrane is compromised (Denk et al., 1992). In many instances following aminoglycoside treatment, morphological assessment reveals that hair cells are actually fused to one another (Arritola, 1995).

Aminoglycosides can inhibit phosphoinositide metabolism in cell membranes and can interfere with the calcium binding sites in the membranes (Stiffler, 1993). The interference with the calcium dependent mechanisms can effect the actin/myosin interaction and can result in the shortening of the outer hair cells (OHCs) of the mammalian cochlea (Cecola and Bobbin, 1992; Harada et al., 1994). Calcium plays an integral role in hair cell metabolism,

cell shape, and cell excitability (Slepecky et al., 1993). Research on mice suggests a reduction in a calcium-binding protein, S-100, resulting in a disruption of calcium metabolism (Tahara et al., 1995). Still further research suggests that formation of free radicals by aminoglycosides leads to the destruction of hair cells (Clerici et al., 1995; Meiteles and Rapheal, 1994; Priuska and Schacht, 1995).

It is known that the half-life of aminoglycosides is approximately five to six times longer in the otic fluid than in the circulating plasma (Arritola, 1995).

Immunohistochemical methods have demonstrated that aminoglycosides can specifically penetrate into the sensory cells (Aran et al., 1993). However, it is not well understood how the drugs are initially absorbed into the endolymph and perilymph (Walker et al., 1990). It is conceivable that an over-accumulation within the stereovilli causes the blockage of the calcium binding sites (Nakagawa et al., 1992; Walker et al., 1990).

Imamura and Adams, (1995) found that after systemic and local injection, aminoglycosides accumulated in several auditory and vestibular hair cells often causing

disruption of cellular components such as calmodulin, S-100, and sodium-potassium ATPase.

Decreased carbohydrate uptake due to the presence of aminoglycosides may be another cause of hearing loss. It has been suggested that certain carbohydrates are important in anchoring stereovilli tip links of hair cells and as well as anchoring stereovilli to the overlaying tectorial membrane (Epstein and Cotanche, 1995; Plinkert et al., 1992; Tilney et al., 1992). If aminoglycosides disrupted the uptake of carbohydrates, normal cell functioning would be compromised. Of course, one must also realize that different aminoglycosides could each produce different results and different levels of severity.

Current Research:

In some animals, hair cells possess the ability to regenerate after auditory trauma. The regenerative capabilities of amphibians are well known. Unlike the hair cells of amphibians, for many years it was believed that the hair cells of both the avian and mammalian cochlea did not regenerate. This was first challenged by several studies indicating the avian cochlea is capable of

regenerating new sensory cells (Chen et. al., 1993; Cotanche and Lee, 1994). Recently, it has been demonstrated that under certain conditions, mammalian sensory epithelium may also have the ability to recover structurally and functionally (Cotanche et al., 1994).

Although not as prevalent as research on amphibians and birds, research has also been focused on fish (Rubel et al., 1991; Corwin et al., 1991). However, like amphibians, teleost fish possess a continual post-embryonic production of sensory hair cells (Weisleder and Zakon, 1995; Presson et al., 1995; Corwin et al., 1991). In fact, a possible correlation has been discovered between the number of hair cells damaged and the size of the fish. Lombarte et al. (1993) state "The time course for damage was longer in larger fish, but the recovery of the ciliary bundles appeared to be complete about 10 days after the maximal damage was seen in both the smaller and larger fish." The drugs were injected intramuscularly and the experimental animals sacrificed post-injection to examine the condition of the hair cells.

In birds, there is evidence to suggest that hair cells can regenerate in chicks but not in the adult chicken. This could result from the fact that the chick

tissue still contains proliferating cells. The hair cells, the production of which normally occurs in embryogenesis, have been found to undergo mitosis after acoustic or ototoxic trauma (Raphael, 1993; Oesterle et al., 1993; Stone and Cotanche, 1992).

Many of the studies on avian hair cells have used pure tones as the source of trauma while others have used various aminoglycosides as the source of trauma (Adler et al., 1992; Li et al., 1994; Lomax et al., 1995; Pugliano et al., 1993b). Aminoglycosides commonly used experimentally include gentamicin, streptomycin, and kanamycin (Duckert and Rubel, 1993b; Weisleder and Rubel, 1993; Hashino et al., 1992). Finally, Lippe et al. (1995) reported that aminoglycoside toxicity not only damages the hair cells but also causes a loss of spiral ganglion cells, i.e., the auditory nerve ganglion. Loss of spiral ganglion cells could compromise hair cell recovery (Lippe et al., 1995).

Most of the research on avian hair cells includes the harvesting of hair cells and microscopic examination post-exposure (Duckert and Rubel, 1993b; Stone et al., 1995). Furthermore, in most instances, the drugs being tested were either injected into the animal or fed to the animal.

Both of these methods may include a degree of uncertainty as to exactly how much of the drug is damaging the hair cells. Very few studies on birds examined threshold shifts using ABR techniques (Lou et al., 1994). Most of these use several different stimuli of different frequencies (Trautwein, 1995). For example, in order to damage the hair cells, chicks were exposed to pure tones. In one particular study (Saunders et al., 1993), chicks were anesthetized, tracheotomized, and hearing examined using ABR methods. Electrodes were inserted near the brainstem and the animals stimulated with tones of various frequencies. In another study using ABR techniques and noise dosing, it was found that after fourteen days, the thresholds returned to their pre-exposure levels (Umemoto et al., 1993). An interesting study involved the training of adult quails to peck when tones were detected. Recovery times were then examined after exposures to pure tone stimuli (Niemiec et al., 1995).

In mammals, research has been focused on monkeys, pigs, chinchillas and rats among others (Alles and Pye, 1993; Ankerstjerne et al., 1995; Canlon et al., 1988; Quint et al., 1995). Limited research has also been conducted on human subjects. Recently, numerous studies

have focused on the proliferation of sensory hair cells in mammals since hair cell regeneration in mammals had been thought to be not possible. However, this has since been proved to be incorrect (Abdouh et al., 1993; Cotanche and Lee, 1994; Forge et al., 1993; Lefebvre et al., 1993; Warchol et al., 1993). Normally, the production of hair cells is completed at the end of the first half of the gestation period. However, repair of stereovilli after damage suggests the capability for replacement of hair cells in the mammalian cochlea (Lefebvre et al., 1993).

Some of the initial research supporting the regeneration of mammalian hair cells was conducted on rats. However, research on guinea pigs and chinchillas is now very common (Pye and Collins, 1991). Lefebvre (1993) found that retinoic acid has the capability to stimulate the regeneration of hair cells in rats following damage to the hair cells by aminoglycoside drug treatment. Other studies have also found a correlation between retinoic acid and hair cell production (Kelley et al., 1993). Few studies examine drug effects or noise dosing effects using ABR methods (Hiel et al., 1993; Ma, 1990). As for birds, much of the research on mammals involves the sacrificing of the animal in order to examine the hair cells

microscopically (Lefebvre, et al., 1993; Meza-Ruiz et al., 1995; Quint et al., 1995).

Research on humans has been limited to subjects receiving drug treatments (Cheung et al., 1990). Of course, neither examination of the hair cells nor single-unit recording is feasible in humans so, ABR methods are utilized. Throughout the drug course for patients receiving aminoglycosidic treatment, ABR data was collected for early detection of ototoxicity (Fausti et al., 1992; Salonna et al., 1992). This study not only provides valuable data for humans, but can also be used to help determine safe therapeutic levels before the ototoxicity or nephrotoxicity occur (Shrimpton et al., 1993).

Anatomy of the Frog:

Even though there are similarities between the mammalian and anuran ear, there are major external ear and inner ear differences. While anuran amphibians do not have a pinna, most possess tympanic membranes contiguous with the skin. Rather than three middle ear ossicles, there is usually a single columella which mechanically links the tympanum to the inner ear fluid space. Anurans

also have two auditory organs, the amphibian papilla (AP) and the basilar papilla (BP) (Wilczynski and Capranica, 1984). The AP is sensitive to low frequencies in the range of 100 to 1500 Hz while the BP is sensitive to higher frequencies in the range between 1500 and 5000 Hz (Duellman et al., 1986). These ranges often vary from species to species and sometimes between sexes. The AP is generally larger than the BP. The AP hair cells possess both efferent and afferent synapses. The BP receives only afferent fibers (Flock and Flock, 1966; Simmons et al., 1992; Sobkowicz and Slapnick, 1994). Like birds, new hair cells are produced at a low level throughout life in amphibians (Kelley et al., 1992).

Research on Frogs:

Even though much has been studied about ototoxicity and regeneration in the auditory system of various animals, much of the focus has been on mammals, birds, and fish. Of the smaller amount of research carried out on amphibians, most studies have examined bullfrogs, *Rana catesbeiana* (Hernandez et al., 1995; Northcutt and Ronan, 1992; Seaman, 1991). Some of these studies have examined the effects of aminoglycosides on the morphology of

auditory hair cells (Baird et al., 1993; Kroese et al., 1989). The present study is unique in being the first to examine, albeit indirectly, the physiological consequences of aminoglycoside treatment on the frog auditory system. This is also the first study to use high frequency and low frequency clicks to evoke specific amphibian or basilar papilla responses.

Methods and Materials

The leopard frog *Rana pipiens* (Charles D. Sullivan Company, Nashville, TN) was utilized exclusively for this study. All animals were medium size adults (2 1/2 - 3 inches). All conditions and environments were standardized and kept constant. After shipment, the frogs were promptly placed in 20" x 10" tanks (usually five per tank). The temperature varied between 27-30 degrees Celsius. Approximately five crickets were fed weekly to each frog starting on their day of arrival. The mean mass \pm standard deviation of the frogs was 43.3 \pm 5.4 grams. Each test animal underwent surgery for electrode implantation between 3-10 days after arrival. After the surgeries and for the duration of testing, the frogs, in groups of three, were transferred to 8" x 16" tanks.

The frogs were implanted under anesthesia (TMS, 3.0 mM, bicarbonate buffered) and placed dorsal side up on ice during the surgery to slow blood flow. The electrode implants consisted of three wires which performed the

following functions: active-1, active-2 and reference. Each electrode consisted of a 2.5 cm length of teflon-insulated seven-strand stainless steel wire (.15 mm in diameter, A-M systems). Approximately 10.0 mm of the insulation was removed from one end of the wire for amplifier connection. The implanted end of the electrode was stripped of 0.5 mm of its insulation. The active-1 electrode was placed within the cranium near the cochlear nucleus, the active-2 near the dorsal lymphatic sac, and the reference placed medially, between the eyes. The active electrodes were placed so as to detect the largest possible BAEP (Brainstem Auditory Evoked Potential). This placement was determined by trial and error. A 25 μ m diameter drill bit was used to drill three holes through the cranium to provide access to the brain. Once positioned, the electrodes were sealed in place with cyanoacrylate adhesive (Nexaband Ophthalmic) and dental cement. Once dried, the electrode cap and electrode bases were coated (Plasticdip) for added stability. For identification of the frogs a color code system was utilized. A combination of three colored beads was allowed to dry in the electrode cap created by the dental cement and Plasticdip. Animals were allowed 48 hours

minimum recovery before testing. Commonly, the frogs exhibited normal behavior within 12 hours following the surgery. The implants usually lasted from three to six weeks. Each experiment was performed in a sound isolated room (IAC 1200 series).

The sound producing equipment consisted of a computer controlled "click" generator (Macintosh, GW instruments), equalizer (Rane FPE 13 Parametric), amplifier (Hafler P230) and loudspeaker (Radio Shack Minimus 7.0). Figure 1 shows the stimulus and recording equipment arrangement. The speaker was hung one meter directly above the test-frog. This arrangement enabled both tympani of the frog to be stimulated equally. The signal recording equipment consisted of signal amplifiers (Precision Instruments DAM 80, Tektronix 26A2) and a Digital Signal Analyzer (Tektronix DSA-601A). The amplifiers were bandpass limited (1 Hz - 3000 Hz). Using this arrangement, the brain signal was amplified approximately 10000x. The sound from the speaker at the position of the test animal was monitored using a measurement microphone and filter/amplifier (ACO Pacific and Krohn-Hite 3700).

The averaging process was sophisticated in that a copy of the brain signal was routed to a custom-designed "artifact reject" circuit. This circuit validated the trigger pulse for averaging following each presentation of a stimulus click. Validation occurred only if the brain signal remained within a preset limit adjusted to be lower than muscle-generated artifacts present during any locomotor behavior. The circuit effectively looked back in time to examine the incoming signal from the brain. In this way, transient movement of the frog did not impair the on-going average and much time was saved during testing. If the brain signal contained too much artifact, from frog movement for example, the signal would not be averaged. Typically, between 250 and 500 averages were required, depending on the signal/noise ratio for the animal.

My method of determining thresholds for frogs utilized a visual detection method (Carey and Zelick, 1993) with a variation (step size) of the method of adjustment technique. This process involved starting with a loud click (20 dB attenuation, 94 dB SPL) and increasing the attenuation in large steps (20, 10, 5 dB attn) (decreasing the dB SPL level) until no ABR was

seen. When no ABR was present, the attenuation was decreased until the ABR was once again present. Thus, a combination of over- and under-shooting was used in order to determine the threshold point.

Since the aminoglycosides may affect the amphibian and basilar papillae differently, specific clicks were generated so as to stimulate the AP, BP, or both. Thus, the frogs were exposed to three different test stimuli (Figure 2-5). The first of the clicks was a single-period 1 kHz sinewave click. This click was generated from a waveform generator (TDT WGI) and for abbreviation is referred to as the "WGI" click. This click encompassed a wide band of frequencies sufficient to stimulate both the AP and BP while the two other clicks, a low frequency and high frequency click, were specifically designed to stimulate the amphibian and basilar papillae, respectively (Figure 6-8). A pulse generator (Krohn-Hite 2100) was used to trigger both the computer generated clicks as well as the waveform generated click at a rate of 11 per second which was found to be optimal in a previous study (Carey and Zelick, 1993). The computer generated clicks are referred to as the "AP" and "BP" clicks.

The AP and BP clicks were actually composites of multiple sine waves (over the desired range). The sine waves were of coincidental phase at the middle of the click. Given the limitations of the computer and the mathematics involved, approximately 24 Hz steps were utilized between sinewaves. For example, if the first frequency was 200 Hz, the second would be 224 Hz, followed by 248 Hz, and so on. The composite sinewaves produced a "train" of pulses as a consequence of falling in and out of phase with each other (constructive and destructive interference). A single pulse was sliced out of the "train" and further refined by applying a Hanning Window (cosine squared wave). This produced a click of the desired duration and spectral composition.

Initially, all of the frogs underwent sound testing to establish base-level "normal" ABRs for all three clicks and to determine the thresholds for each frog. Each frog was utilized as its own control in subsequent tests. Approximately six days of determining thresholds were needed per frog in order to establish each frog's normal thresholds. After normal thresholds were obtained, each test animal was injected with gentamicin sulfate (Sigma). Unlike several other studies on birds,

fish, and amphibians in which gentamicin was injected systemically, a bilateral intraotic administration of gentamicin was utilized (Baird et al., 1992; Weisleder and Rubel, 1993; Lombarte et al., 1993). This method eliminated the possibility of systemic overdosage resulting in toxicity and other side effects. In addition, the intraotic administration permitted more certain control of dosage and destination of the gentamicin.

Using the intraotic approach required an estimation of the mean otic capsule volume in order to establish the final concentration of the gentamicin within the otic capsule (Table 1). This is the first time gentamicin was injected intraotically in this frog species. This also provides the approximate intraotic concentration of gentamicin. Several different frogs were utilized to provide a random sample. Four different methods were utilized to determine the otic capsule volume. The first method consisted of drilling two holes into each otic capsule. Dye was injected into one of the holes. When the dye was displaced from the second hole, the volume of dye injected was recorded. The second method involved drilling one hole into the otic capsule followed by

suction removal of the fluid inside the capsule. The volume of fluid removed was recorded. Both of these methods utilized a Hamilton syringe for precise measurement. The third and fourth method involved the removal of the entire otic capsule. After the connective tissue was scraped away, the dimensions were recorded. The approximate volume was calculated assuming the capsule was (1) cuboidal, or (2) spherical. Averaging all four methods, the volume was estimated to be $16.8 \mu\text{l} \pm 4.3$ (SD). For the drug calculations, $15 \mu\text{l}$ was utilized, to compensate for internal tissue and organs.

The gentamicin solution was dissolved in low-calcium HEPES-buffered saline (118 mM sodium, 2 mM potassium, 118 mM chloride, 4 mM D glucose and 10 mM HEPES dissolved in distilled water). Control frogs were injected with the HEPES-buffered saline alone. Test frogs were injected with the HEPES solution with varying concentrations of gentamicin sulfate. Three different concentrations of gentamicin were utilized. The initial concentrations of $750 \mu\text{M}$, $1125 \mu\text{M}$ and $1500 \mu\text{M}$ were injected to yield approximate intraotic concentrations of $200 \mu\text{M}$, $300 \mu\text{M}$ and $400 \mu\text{M}$ respectively (see appendix for calculations).

The gentamicin received from Sigma contained components of C1, C2 and C1a with molecular weights of 477, 466 and 449 respectively. An average of 464 grams/mole was utilized (Kroese et al., 1989).

The injections were made bilaterally into the otic capsule under TMS anesthesia. To gain access to the otic capsules, frogs were placed ventral side up with the jaw held open using padded paperclips. This also kept the tongue away from the incision area. The connective tissue and muscle was scraped away in order to expose the otic capsule. A drill (45 μ m diameter) was used to perforate the capsules. Two holes were drilled per capsule. This allowed the excess fluid to be displaced. A Hamilton syringe (10 μ l) was introduced into one of the holes and four microliters of the solution was carefully injected. The holes were covered with dental cement and the frogs were allowed to recover overnight. Testing for thresholds was continued the following day until the thresholds returned to the frogs' normal values.

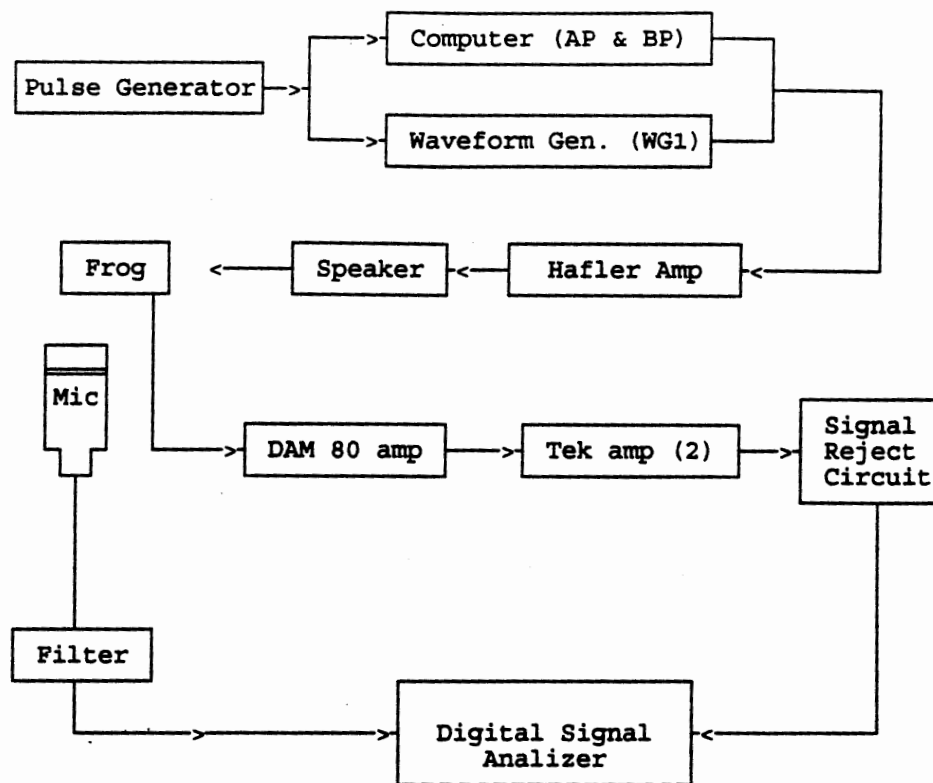


Figure 1. Stimulus and recording arrangement for obtaining ABRs. The clicks, generated by the computer and waveform generator, are triggered by the pulse generator. The clicks are amplified to drive the speaker. The brain signals are amplified and any artifact present is rejected by the signal reject circuit. The signal is averaged and viewed with the digital signal analyzer. The microphone is used to monitor the clicks.

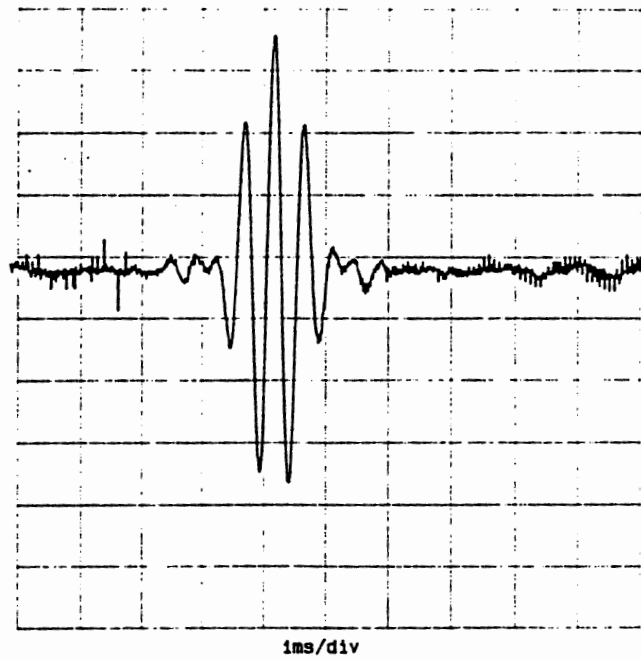
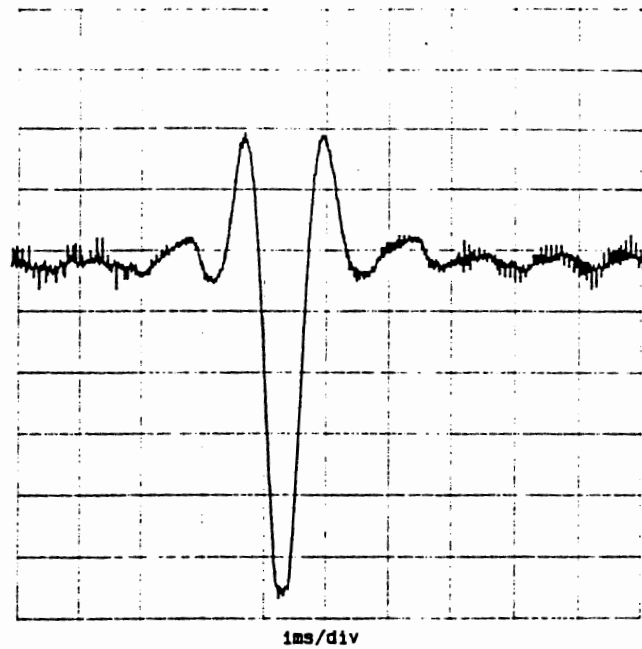


Figure 2. Electrical representations of the AP click (top) and BP click (bottom). Note durations are less than 3 ms. The Y-axis is arbitrary voltage units.

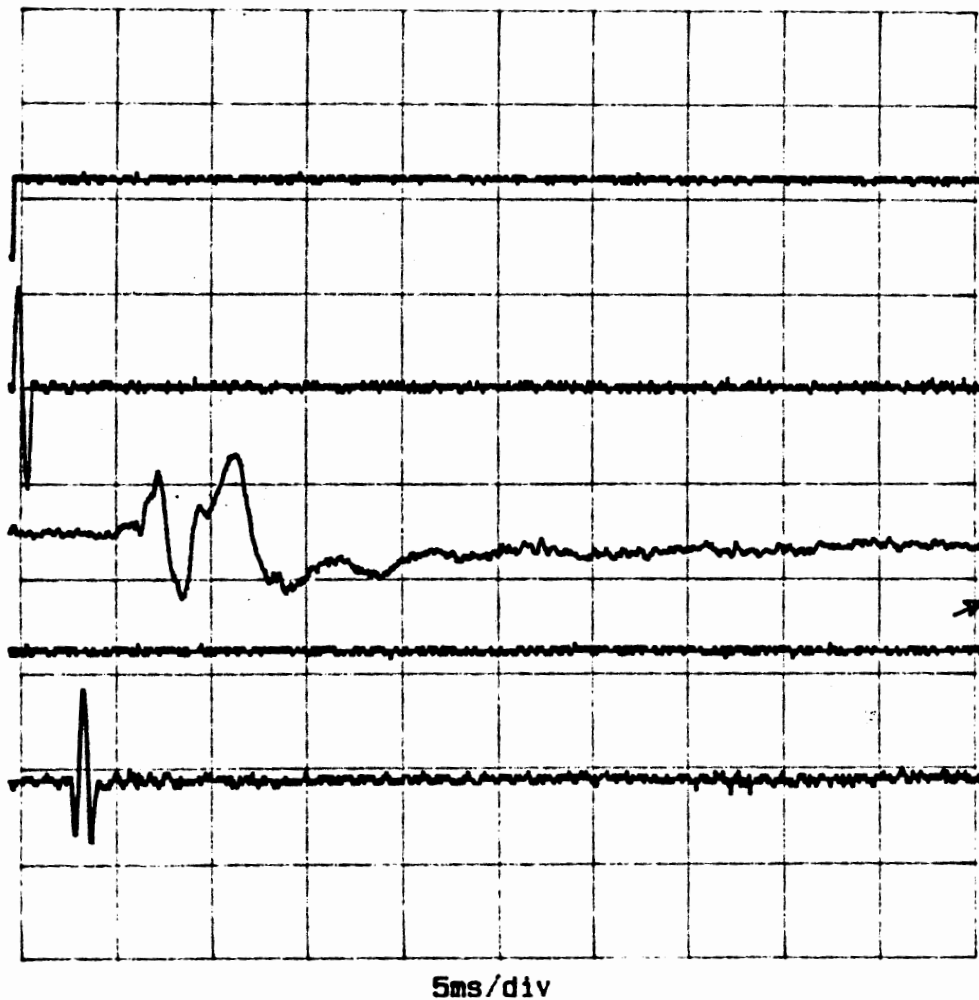


Figure 3. Time relationship of test signals. The top trace shows the triggers for the click generator which produces a sinewave (second trace). The third trace is the resulting ABR. The fourth trace is the validation signal (arrow; see methods). This is also triggered by the main trigger (top trace). The bottom trace is the acoustic signal picked up by the microphone. Notice the delay between the original click, the microphone, and the ABR signal produced. The Y-axis is arbitrary units.

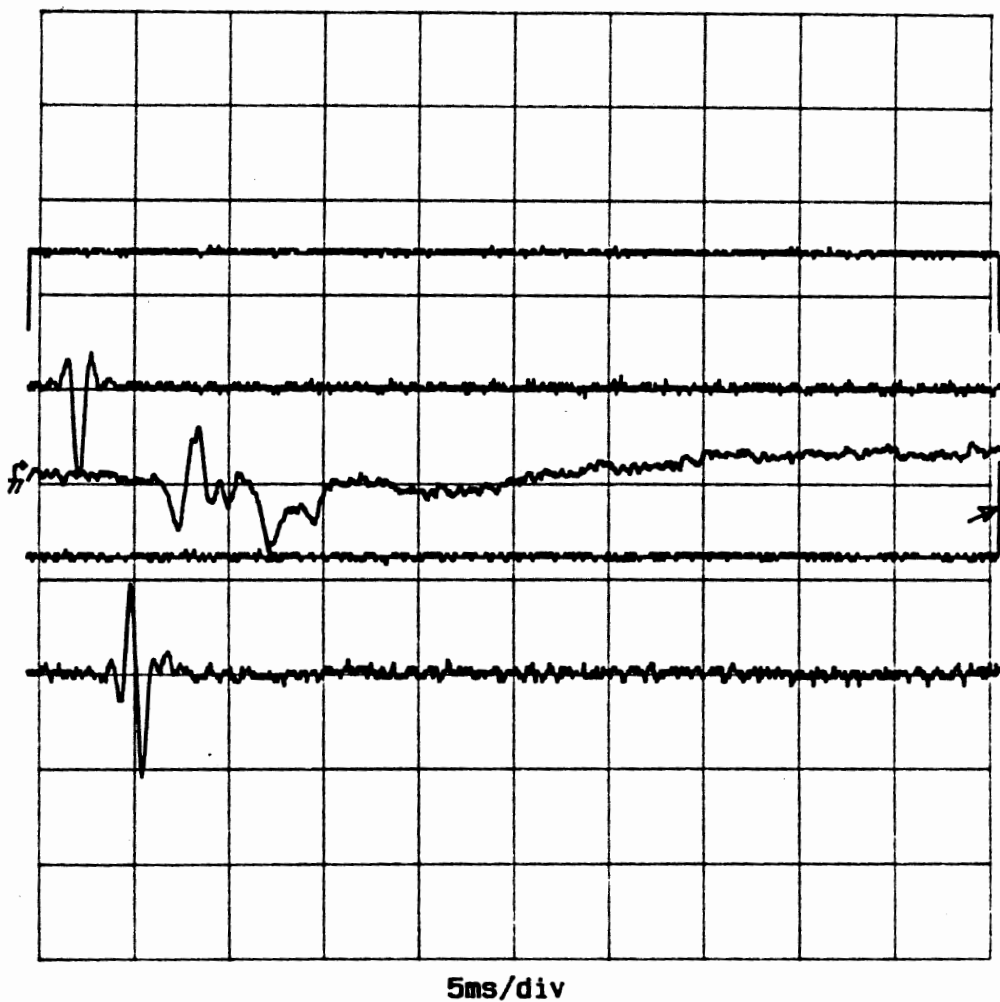


Figure 4. Similar to the previous figure (Figure 4), the trigger (top trace) causes the computer to generate a click, here, the AP electrical signal (second trace). The ABR waveform morphology (third trace) is different then that of the WGI ABR morphology. The fourth trace represents the validation signal. The last trace is the microphone signal.

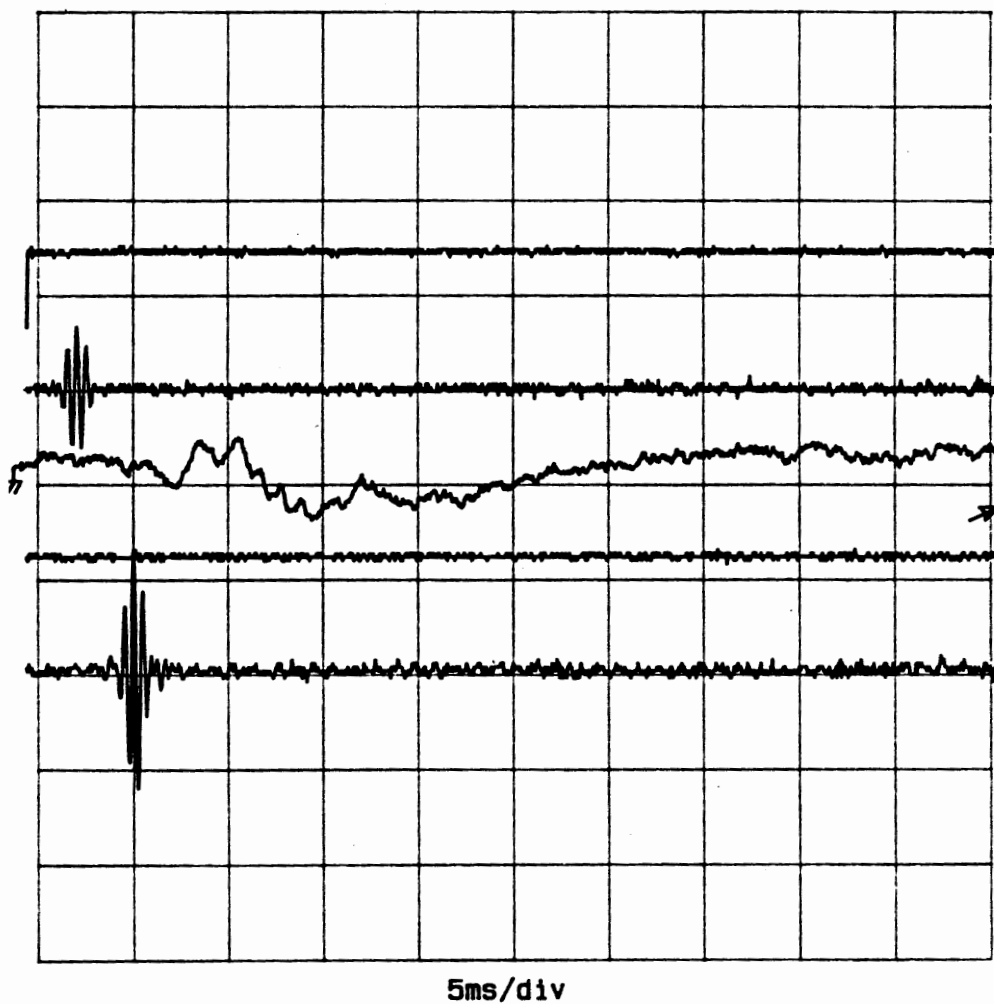


Figure 5. BP click. Traces same as Figure 4. The ABR (third trace) has a different waveform morphology than the WGI or AP clicks.

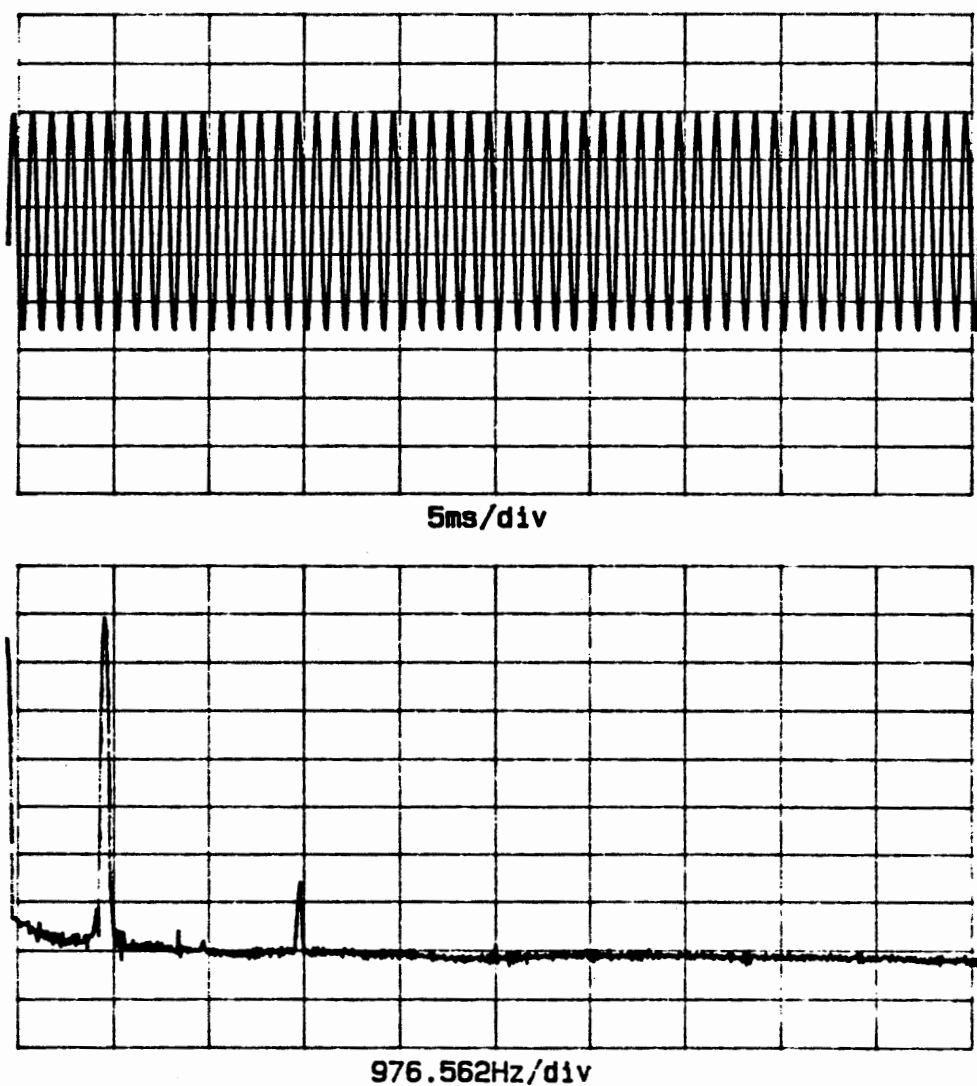
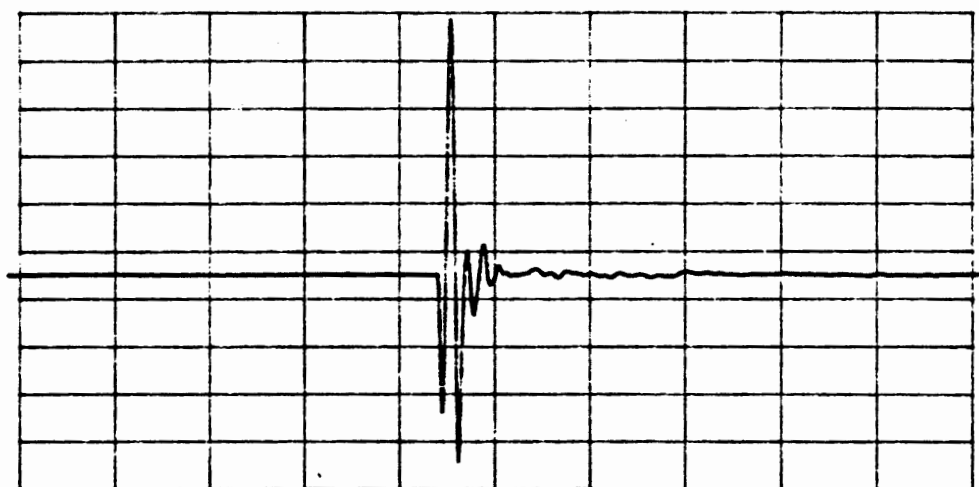
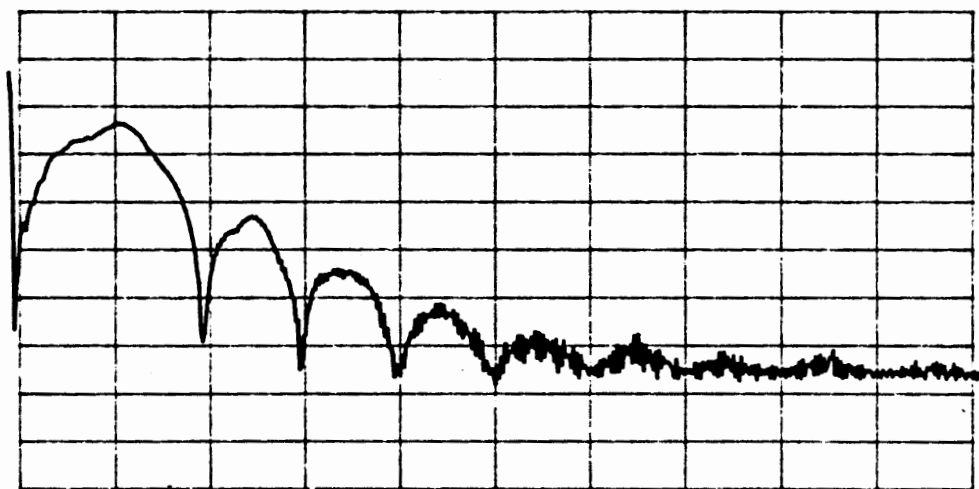


Figure 6. Spectral purity of sound delivery system. Top: A continuous sinewave tone (1000 Hz). Below: The corresponding frequency spectrum. The small peak at 3000 Hz is the 2nd harmonic. The frequency spectrum was averaged 512 times. All Y-axis units are arbitrary units.



5ms/div



976.562Hz/div

Figure 7. Top: A single period sinewave click (TDT click). Bottom: The corresponding frequency spectrum. This spectrum (averaged 512 times) is normal and characteristic of such a click. All Y-axis units are arbitrary voltage units.

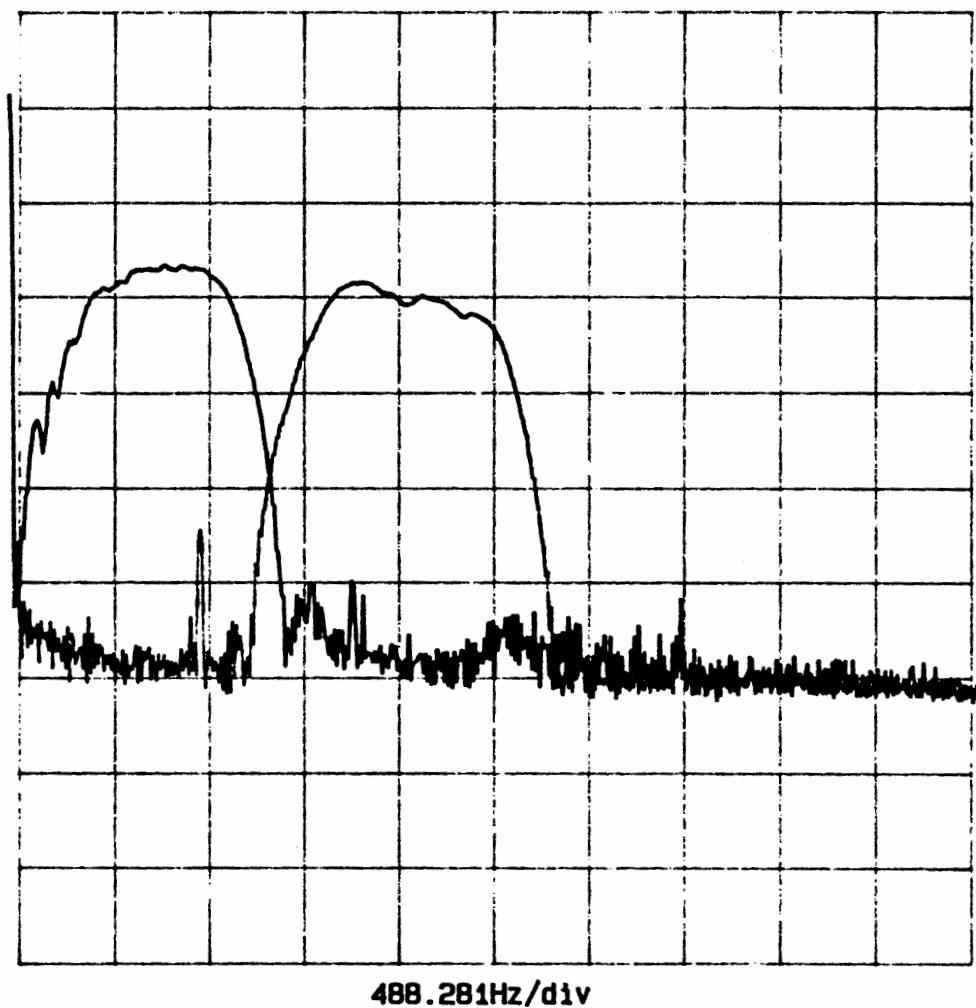


Figure 8. Frequency spectra of the AP and BP acoustic signals. The AP click has most of it's energy between 200-1100 Hz while the BP click mainly contains frequencies between 1100 -2200 Hz. A small overlap is also visible between 1000 and 1100 Hz. Left edge of X-axis is approximately 0 Hz. Y-axis is 10 dB/division.

Method I - Injected Ink

| Frog # | amt. injected |
|-----------------|----------------------|
| Frog 1 | 16 |
| | 15 |
| Frog 2 | 18 |
| | 19 |
| Frog 3 | 14 |
| | 13 |
| Frog 4 | 13 |
| Frog 5 | 25 |
| Mean: | 16.6 |
| St. Dev: | 4.0 |

Method II - Suction Removal

| Frog # | amt. removed |
|-----------------|---------------------|
| Frog 1 | 18 |
| | 17 |
| Frog 2 | 13 |
| | 15 |
| Frog 3 | 20 |
| Mean: | 16.6 |
| St. Dev: | 2.7 |

Method III - Calc. of Sphere

| Frog # | Otic Capsule Volume |
|-----------------|----------------------------|
| Frog 1 | 12.1 |
| | 7.8 |
| Frog 2 | 14.7 |
| | 14.6 |
| Frog 3 | 11.1 |
| | 9.7 |
| Mean: | 11.7 |
| St. Dev: | 2.7 |

Method IV- Calc. of Cube

| Frog # | Otic Capsule Volume |
|-----------------|----------------------------|
| Frog 1 | 23.1 |
| | 14.8 |
| Frog 2 | 28.1 |
| | 27.9 |
| Frog 3 | 21.2 |
| | 18.6 |
| Mean: | 22.3 |
| St. Dev: | 5.2 |

Mean of Method I-IV: 16.8
Standard deviation of Method I-IV: 4.3

Table I. The four methods for the estimation of the otic capsule volume.

Results

ABRs:

ABRs from a typical single frog recorded from pre-treatment through 14 days post-treatment are shown in Figures 9-12. The addition of gentamicin has an obvious effect on the ABR morphology. Absolute peak latencies shift accordingly with the changes in sound pressure level (SPL). With a decreased SPL (increased attenuation), the latencies increased and the amplitude of the ABRs decreased. The corresponding threshold shifts are plotted (Figure 13 top). The ABRs showing the waveform morphology changes correspond to two days before treatment and three, 10 and 13 days post-treatment. This test included only the WGI click. A typical frog showing threshold shifts for injections of 300 μM gentamicin concentrations is also shown (Figure 13 bottom). These data include threshold shifts for the AP and BP clicks in addition to the WGI click.

Threshold and Recovery:

Figure 14 shows the control and mean of frogs receiving 200 μM gentamicin concentrations. The time scale does not completely correspond to that in the other trials since the control frogs were injected with the HEPES-buffered control solution five days prior to the test frogs. As expected, the control group showed little change in threshold. The mean threshold shift for frogs receiving 200 μM gentamicin injections was approximately five dB. Only three frogs were used for the 200 μM injections. Because the shift was small and had no effect on one of the frogs, it was decided to increase the concentration to 300 μM . Baird et al. (1993) found that this concentration of gentamicin had little or no effect on the morphology of frog vestibular hair cells.

Two different groups of frogs received 300 μM gentamicin injections (Figure 15-16). However, only one control group was utilized which showed no significant threshold change. A total of 12 frogs was utilized for the 300 μM trials. The mean threshold shift for frogs receiving 300 μM gentamicin injections was between 5-10 dB.

In frogs receiving 400 μM drug treatments, threshold shifts of 10-20 dB were observed (Figure 17-18). More than 20 frogs were utilized for the 400 μM trials. The first trial of 400 μM injections produced pronounced threshold shifts for which total recovery was not observed. This is attributed to high frog mortality.

Unlike their responses to other drug concentrations, erratic frog behavior was noted for the first time with the 400 μM concentration of gentamicin (first trial only). A total of eight frogs was utilized for trial one, six test animals and two controls. Two frogs, including a control frog died on the third day. On the fifth day, another test frog died. Before they died, all of the frogs appeared "sickly." They also exhibited balance problems and were leaning to one side. Often, one leg would be outstretched. They did not jump and remained still. This included not trying to jump out of tanks even when the lids were removed, a behavior uncharacteristic for frogs. Before the thresholds could completely recover, most of the frogs died. No adverse vestibular effects were observed with the second trial of 400 μM frogs.

In all trials, threshold shifts were seen as early as one day post-injection. Also, recovery began between day three and five post-injection. In both 300 μM trials and the first trial of 400 μM , threshold increases were seen approximately six days post-injection.

Not only did the threshold shifts depend on the dosage concentration of gentamicin; they were also potency dependent. For example, trial two of the 300 μM (Figure 16) injections utilized yielded a smaller change in threshold than the first trial. The gentamicin solution made for this trial was the same used for the first 300 μM (Figure 15) trial and was two weeks old. The potency effect is also obvious from mean threshold differences between the two 400 μM (Figure 17-18) concentration trials. The gentamicin solution used for the second 400 μM trial was six days old. However, the second 400 μM trial shows significant threshold shifts.

It should be noted that on graphs showing threshold shifts (Figure 14-18), three pre-treatment thresholds (day -3, -2 and -1) were averaged. Thresholds recorded three days pre-treatment were usually stable and represented a "normal" threshold. Each threshold

measurement was normalized by subtracting the measurement from the pre-treatment average. The means for each frog were plotted and standard deviations were calculated for all trials except for the controls and the 200 μM trial (Figure 14 bottom).

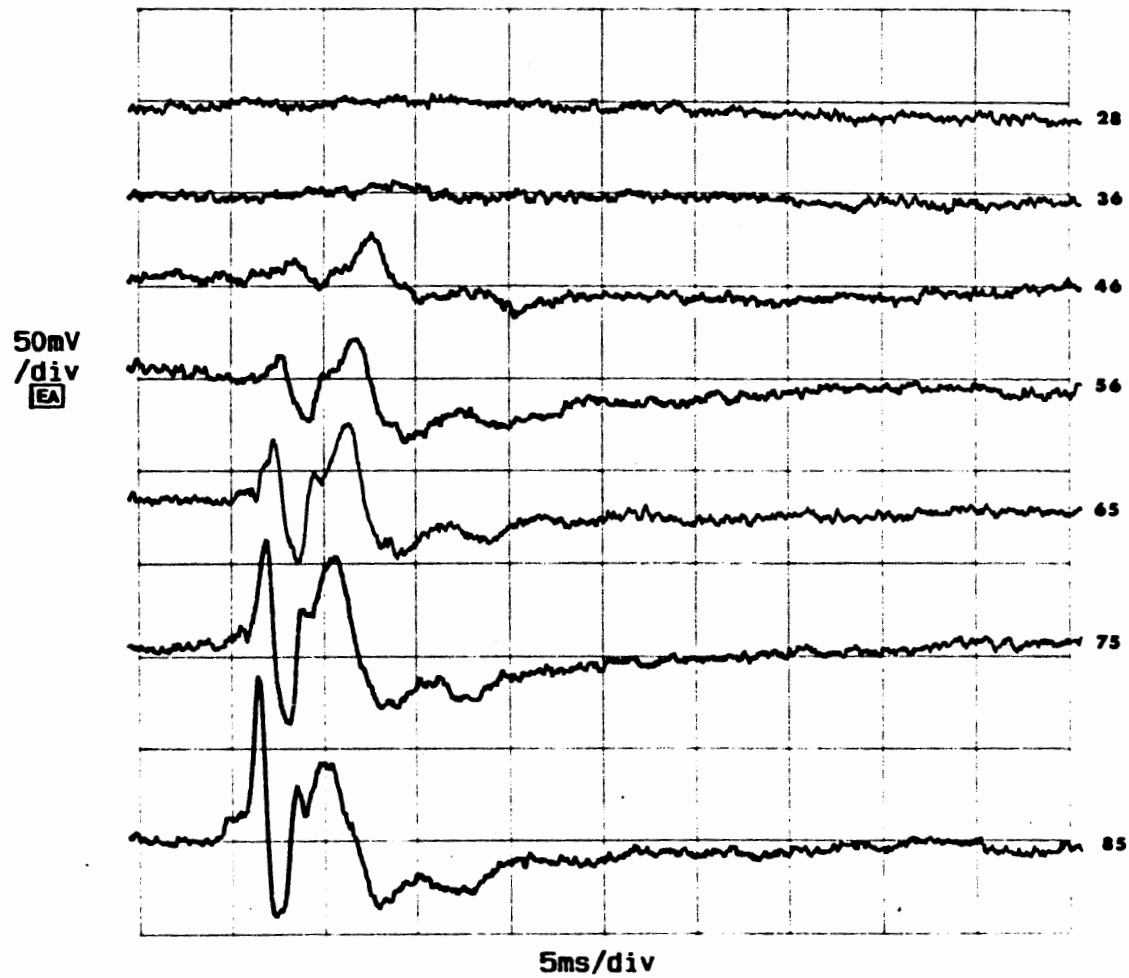


Figure 9. ABR responses recorded two days pre-treatment with gentamicin. Numbers on the right correspond to decibels (dB) SPL.

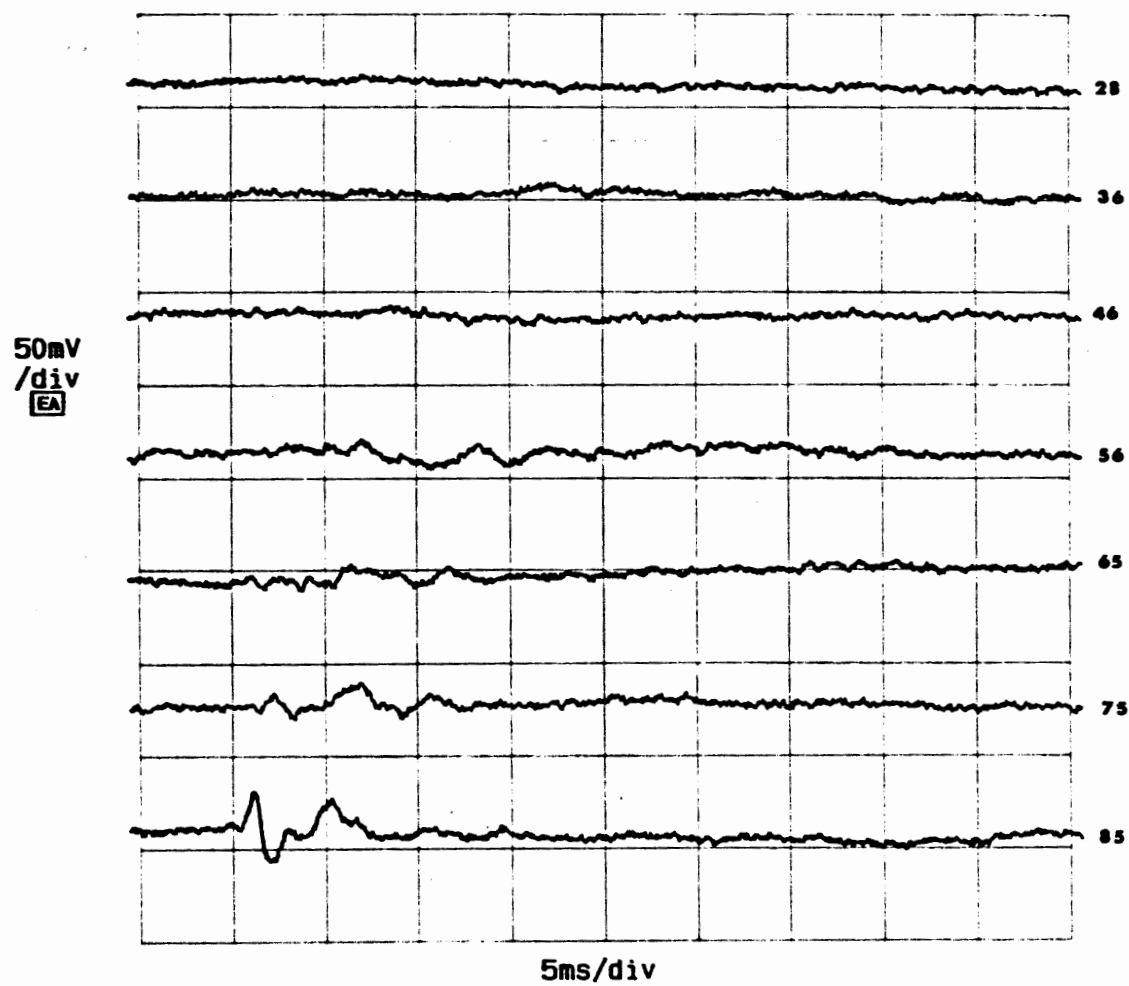


Figure 10. 3-day Recovery of threshold. ABRs from same test animal as Figure 9. Threshold shift is pronounced.

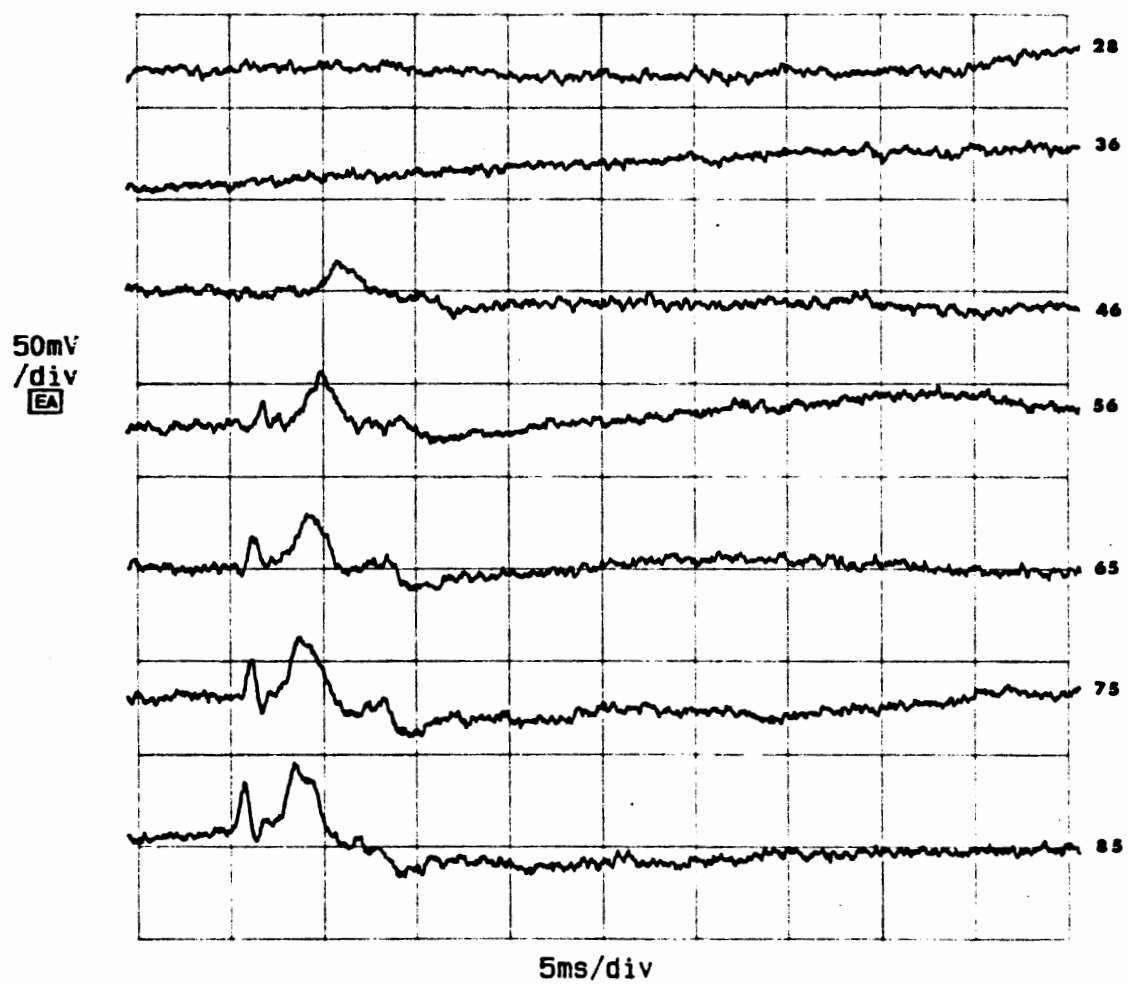


Figure 11. 10-day Recovery of threshold. ABRs from same test animal as Figure 9-10. Note, the waveform morphology is still different.

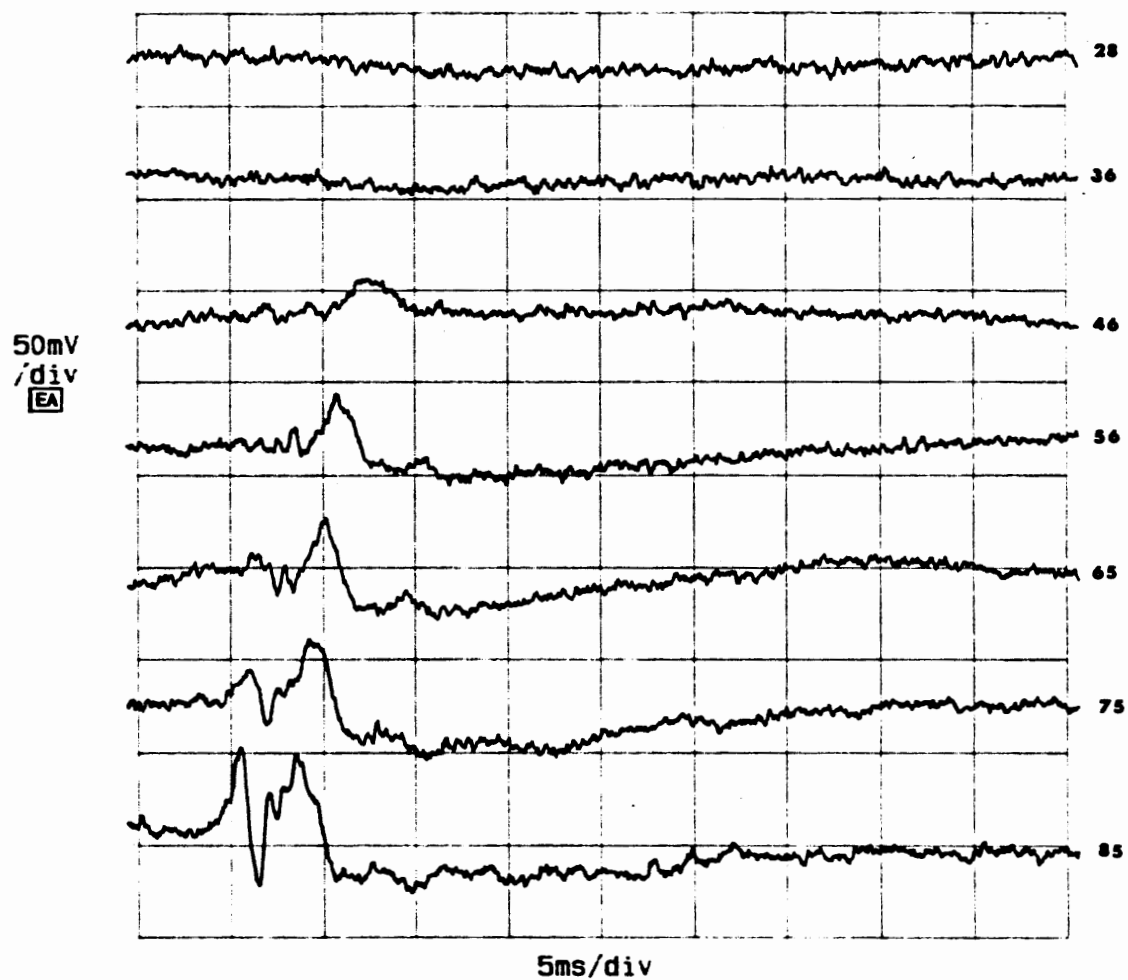


Figure 12. 13-day Recovery of threshold. Both threshold and waveform morphology are similar to the pre-treatment situation.

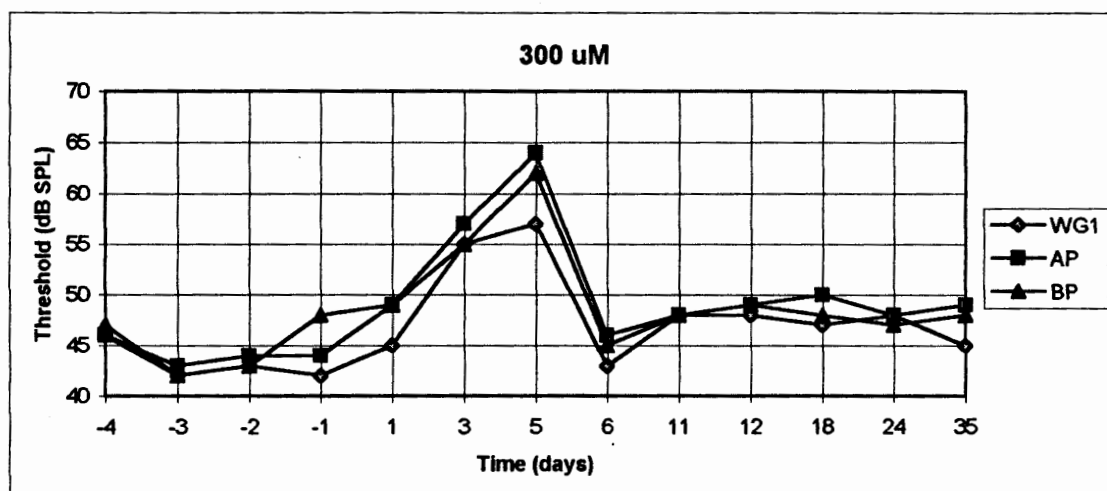
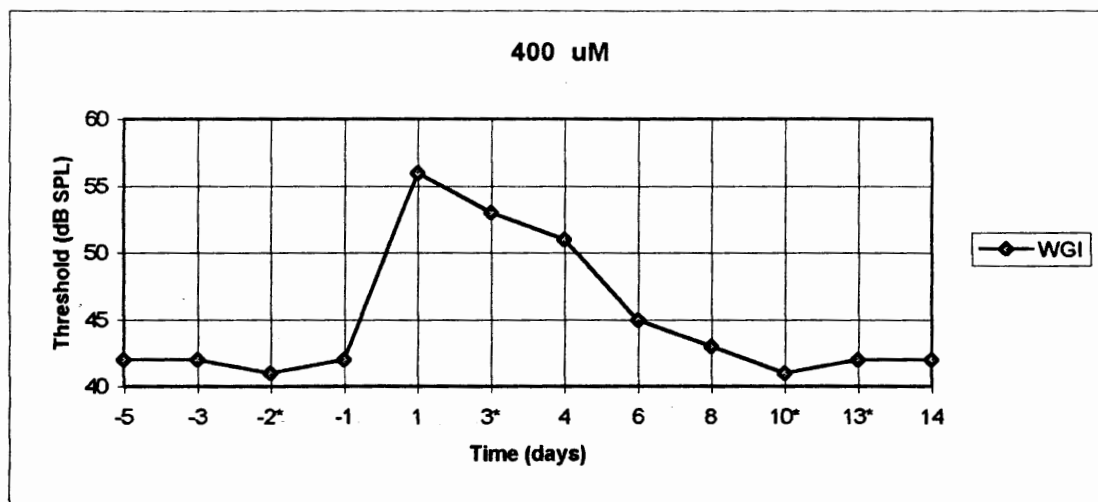


Figure 13. Top: Threshold change and recovery over 20 days. Days marked with an asterisk correspond to ABR data from same test animal as Figure 9-12. Bottom: Typical threshold from one frog receiving 300 μ M gentamicin concentration showing similarity of threshold change for AP and BP.

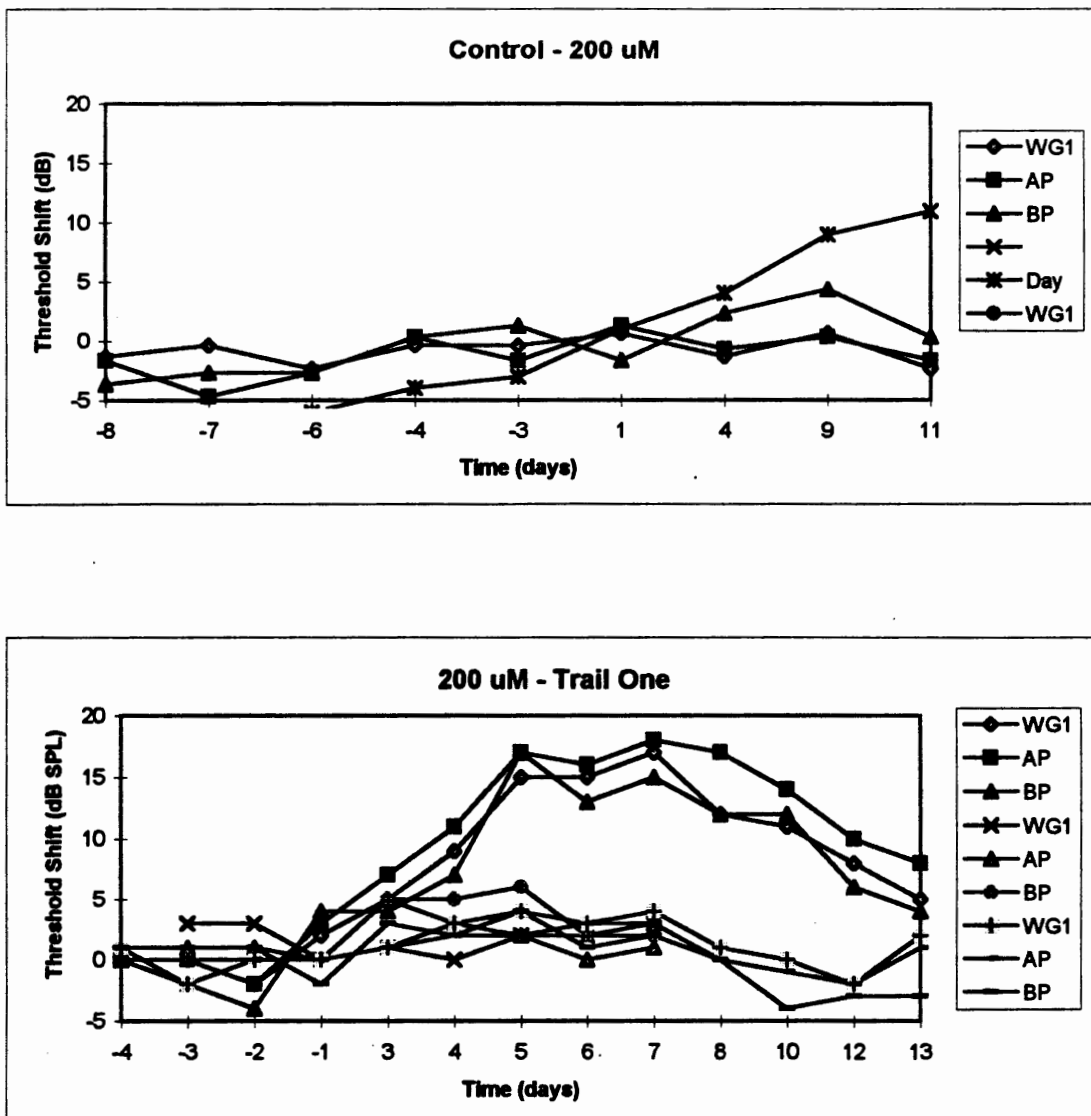


Figure 14. Top: Control group for 200 μ M frogs. Bottom: Threshold shift for three frogs given 200 μ M gentamicin. Control frogs started six days earlier than test frogs.

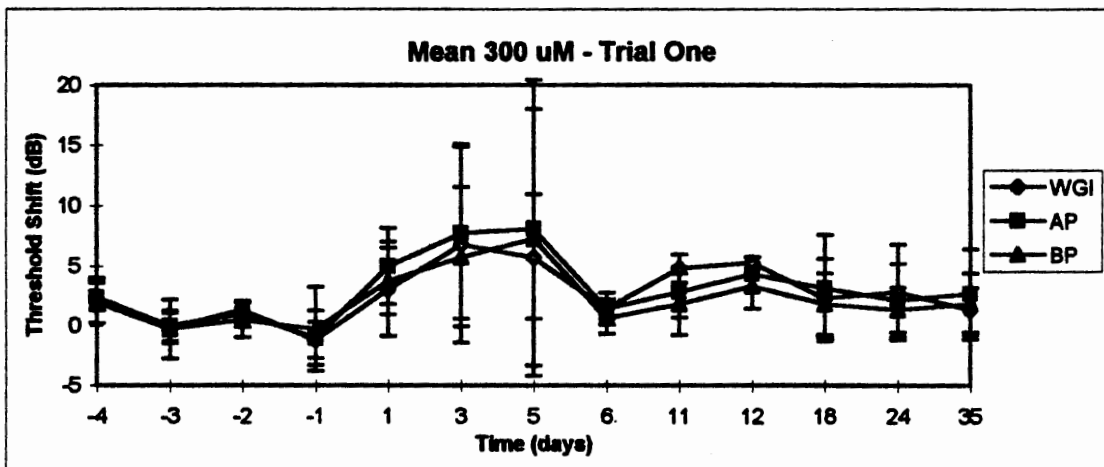
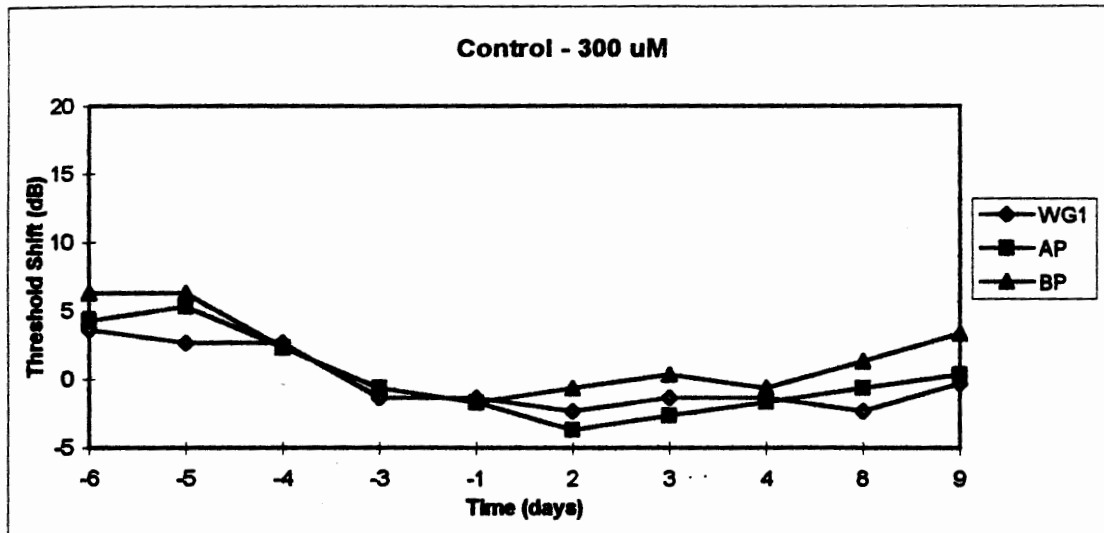


Figure 15. Top: Control group for 300 μ M frogs. Bottom: Trial One threshold shift of frogs given 300 μ M gentamicin. (Mean \pm SD for WGI, AP, and BP clicks, $n=5$). Since only one control group was used for the 300 μ M injections, the control groups are the same for Figure 15 and 16.

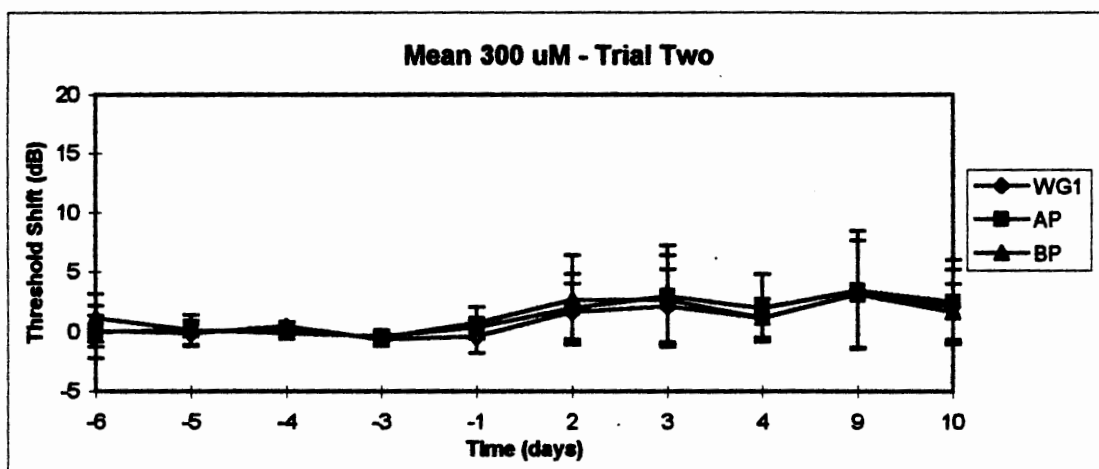
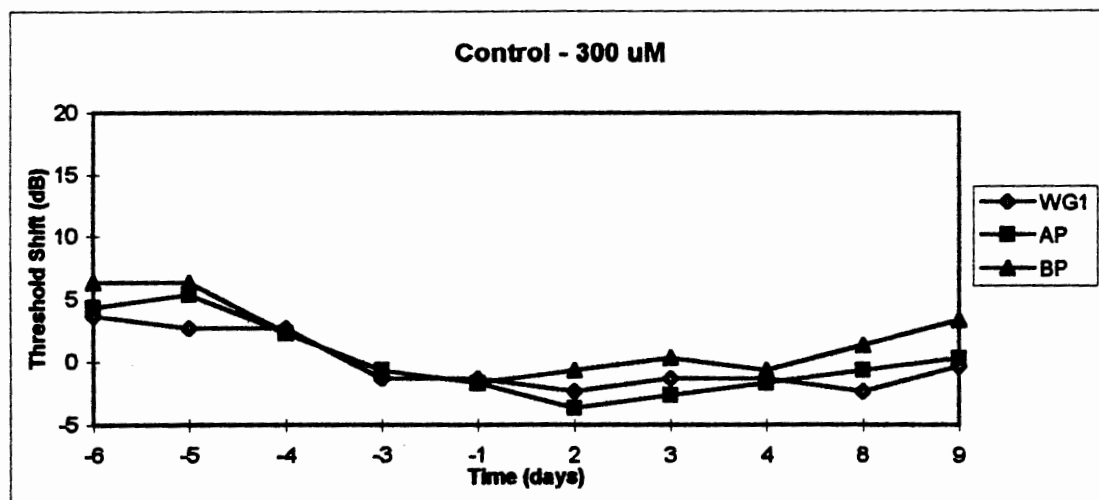


Figure 16. Top: Control group for 300 μ M frogs. Bottom: Trial Two threshold shift of frogs given 300 μ M gentamicin. (Mean \pm SD for WG1, AP, and BP clicks, $n=6$).

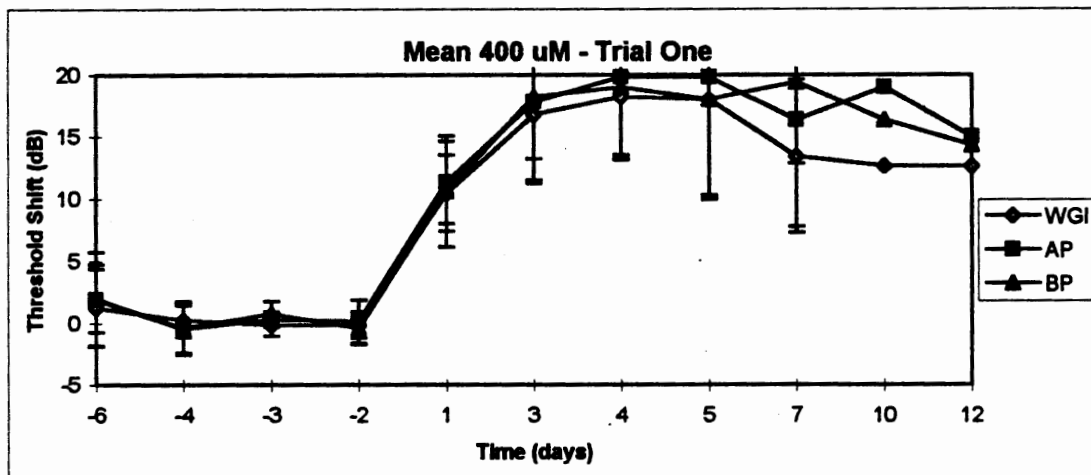
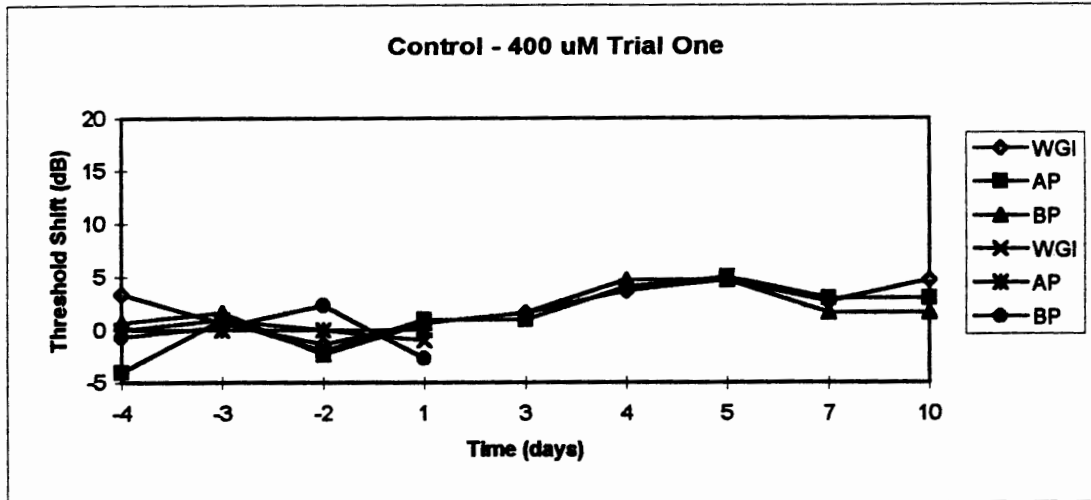


Figure 17. Top: Control group for 400 μ M frogs. Bottom: Trial One threshold shift of frogs given 400 μ M gentamicin. Threshold shifts were between 15-20 dB. However, high mortality rates and suspected infection prevented return to “normal” thresholds. (Mean \pm SD for WGI, AP, and BP clicks, n=8).

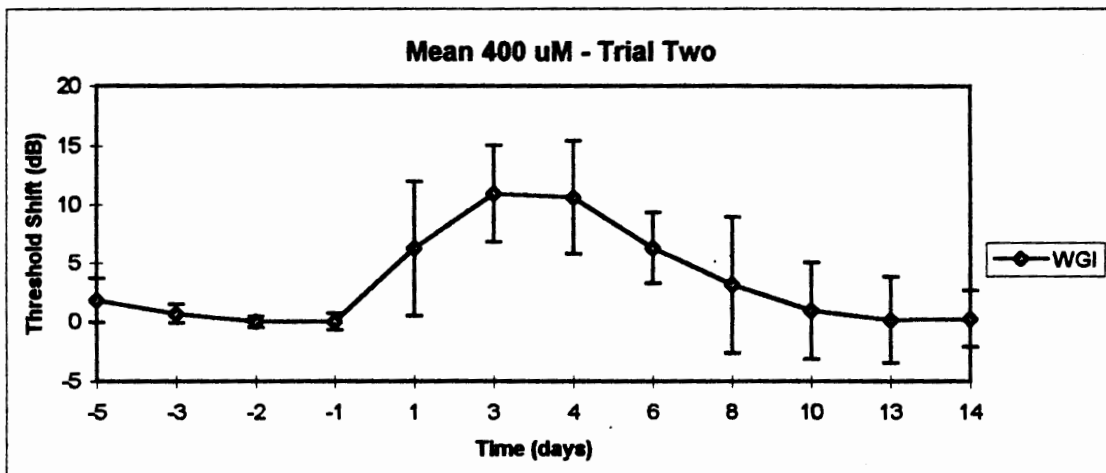
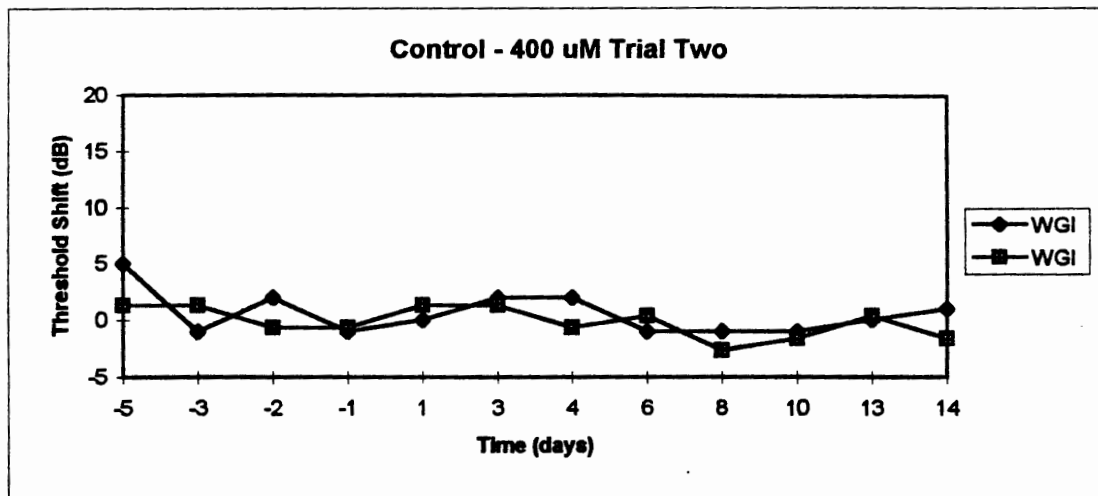


Figure 18. Top: Control group for 400 μ M frogs. Bottom: Trial Two threshold shift of frogs given 400 μ M gentamicin. (Mean \pm SD for WGI, AP, and BP clicks, n=10).

Discussion

The evoked potential or ABR technique can be used as an assay for the performance of the ascending auditory pathway. The ABR technique in frogs allows long-term recording of auditory function. I found that it is important to wait at least 24 hours following implantation of electrodes before threshold measurements are recorded. Although the animals appeared awake and active only five hours post-implant, the thresholds were found to be higher than those measured subsequently. Occasionally, even 36 hours after frogs were implanted, thresholds would be higher than normal. For this reason, thresholds were monitored for three to five days to be sure stable low thresholds were obtainable. Figure 15 and 16 (top), the 300 μM control averages, demonstrate how a gradual threshold decrease occurs with time.

Aminoglycosides caused presumed destruction of hair cells as early as one day post-treatment, with a maximum effect observed between three and five days. Often, total recovery is seen between 14-20 days in frogs and birds (Baird et al., 1993; Chen et al., 1993; Hashino et al.,

1991). In my study, the same time course of effect existed for the threshold shifts observed in all trials. Unfortunately, threshold recovery within the two week time period did not occur with all frogs because several frogs sickened and died. Even though Baird et al. (1993) found that bullfrogs (*Rana catesbeiana*) did not survive 400 μ M injections, I believe that an infection was to blame for the high frog mortality initially observed with *Rana pipiens* given a similar dose. This is supported by the observation that a control otic puncture frog also died. In order to combat any infections that might have affected the frogs, prophylactic antibiotic (non-aminoglycosidic tetracycline) treatments were administered in later experiments. Mortality and sickness decreased markedly as seen with the second trial of 400 μ M frogs. In fact, the frogs showed no signs of sickness, balance problems, or lack of activity. Of the trials conducted before the tetracycline treatments, frog thresholds often never reached normal threshold due to sickness and death.

Vestibular toxicity due to aminoglycosidic treatment leads to behavioral changes associated with balance deficits (Honda et al., 1991; Tsue et al., 1994; Walker et al., 1990). In frogs, the observations include

instability of the head and consequent deterioration of jumping ability. Other behaviors noted were abnormal posture and swimming (Suzuki et al., 1991). Research on rats after aminoglycosidic treatment documents vestibular deficiencies ranging from diminished swimming ability to prolonged latency for righting responses (in air) and resurfacing in water, compared to controls (Ruiz-Meza et al., 1995).

In this study, before the prophylactic drug treatments, several frogs succumbed to a possible infection and died. Abnormal behavior such as balance problems and lack of activity was observed. However, these symptoms were not observed in the frogs that received 200 and 300 μM gentamicin injections. Only the first trial of 400 μM test frogs exhibited these characteristics. Frogs treated prophylactically with the tetracycline following otic puncture appeared healthy through the duration of the experiment.

As discussed earlier, this is the first research to examine the possibility of stimulating only the amphibian or basilar papillae using an AP-selective and BP-selective stimulus. From my data it is clear that substantial threshold differences between the WGI click and the AP and

BP clicks do not exist. This similarity of sensitivity is not consistent with tuning data of Feng et al. (1975). Furthermore, there was no difference observed between the AP and BP in their response to gentamicin (Figure 13-17).

There is variation among animals in the AP and particularly BP frequency range (Duellman et al., 1966; Feng and Shopner, 1981; Hetherington, 1992; Megela and Capranica, 1981). Thus, it is possible that the frequency composition of the clicks was such that they were not specific enough to evoke responses exclusively from one or the other papilla. If however, there is truly no difference in drug response by the two papillae, this suggests that any differences in AP and BP hair cells, and the organs themselves, do not confer relative protection from aminoglycoside trauma. What are the differences between the AP and BP? They both lie in their own chamber and are separated. Lewis et al. (1982) found that even though both papillae are innervated by the eighth nerve, axons in the AP connect 10-15 hair cells while in the BP one axon innervates one hair cell. The AP hair cells are specifically tuned to frequencies in the range from 100 Hz to 1000 Hz while the BP is tuned to higher frequencies (Schmitz et al., 1992). However, it should be noted that

the data of Schmitz et al. (1992) on characteristic frequencies were based on pure tones, not clicks. As in mammals, the AP has tonotopic organization. Even though these organs are very different in morphology, it appears the gentamicin can affect both equally. If indeed, as several studies indicate (Aran et al., 1993; Nakagawa et al., 1992; Stiffler, 1993; Tahara et al., 1995), aminoglycosides alter binding sites on plasma membranes leading to disruption of normal cell metabolism, then we can conclude that despite various physiological differences between hair cells, the membranes of all types share sufficient similarities resulting in similar aminoglycoside effects.

A further consideration is the fact that the AP and BP clicks produced ABRs with different waveform morphology. The only effect gentamicin appeared to have on the ABRs from all three clicks was a reduction of amplitude. Characteristic of a typical frog ABR is four peaks. Like humans, which typically have seven peaks, the exact origin of all peaks in frogs is unknown. The reduction in amplitude of the peaks due to the gentamicin was especially pronounced in the first peak. This is to be expected since the damage is occurring at

the hair cell level. The amplitude of peak I and subsequent reduction is clearly demonstrated in Figure 9-12. Recovery of the hair cells returns peak I to nearly pre-treatment amplitudes. The same phenomenon occurs to a degree with peaks II-IV.

Normally, the waveform morphology is very similar for the WGI and AP click. This is readily explained since most of the contribution to the overall evoked potential elicited by the WGI click comes from the AP, the organ with more hair cells. Typically, peaks I-IV are all present and approximately the same amplitude. However, the BP response differs in that the peaks are often less defined and have slightly smaller amplitudes. In addition, the distance between peaks, interpeak latency, can be longer with the BP waveform.

There may also be morphological differences between the actual hair cells that may determine the ototoxicity from the aminoglycoside. Baird et al. (1993) found that within the vestibular organs, hair cells in the central regions are more sensitive than hair cells in the peripheral regions. He also found that hair cells of the different auditory organs differ significantly in the hair bundle morphology in different membrane regions. Thus, it

is possible that the number of stereovilli and the size of the kinocillium may determine a particular hair cell's susceptibility to aminoglycosides.

My data has definite correlations with current research on hair cell damage. In bullfrogs, Baird et al. (1993) found that within ten days post-injection, the cellular morphology in the gentamicin-injected animals closely resembled that seen in normal animals. In fact, he observed that the cell bodies of hair cells and supporting cells were more ordered and normal in appearance. In birds, several studies indicate that within 14 days, significant hair cell regeneration had occurred (Chen et al., 1993; Hashino et al., 1992; Weisleder and Rubel, 1993). Forge et al., (1993) report that by seven days post-gentamicin injection in guinea pigs, most of the hair cells in the striolar region were replaced completely. While these studies might support my data, it should be noted that they all involve multiple doses of aminoglycosides. Only the methodology of Baird et al. (1993) involves giving a single dose of aminoglycoside.

Research has demonstrated that some aminoglycosides affect hair cells differently, depending on their location

within the vestibular or cochlear system (Mandell et al., 1990; Tsue et al., 1994; Wanke and Moore, 1991). The effects do not appear to be species specific. For example, hair cells in the vestibular system of humans and anurans are particularly susceptible to gentamicin (Baird et al., 1993; Smith and Swenson, 1990; Weisleder and Rubel, 1993). However, Cotanche et al. (1993) report that the avian, budgerigar, cochlear hair cells are more sensitive to gentamicin than kanamycin (60% vs. 35% damage). Hashino et al. (1992) also found that gentamicin can have a prolonged and progressively more damaging effect on hair cells for several weeks after the end of drug treatment. However, in these avian studies, the aminoglycosides were given in multiple doses over 10 days. Perhaps the damage to the hair cells depends upon the dosing schedule. Smith and Swenson (1990) report that all aminoglycosides will produce cochlear and vestibular toxicity if high concentrations are maintained in the inner ear. Certainly, aminoglycosides given over 10 days compared to one single treatment may affect the vestibular and cochlear hair cells differently. Baird et al. (1993) found that within two days treatment with 200 μ M gentamicin, hair cell density in the sacculus was zero and

in the utricular striola hair cell density was about 25% that of control bullfrogs. This supports the fact that gentamicin affects the vestibular system more than the cochlear system and by the fact that my data shows only a 20 dB reduction in threshold. Thus, not all cochlear hair cells were destroyed since ABRs were still produced even at gentamicin concentrations of 400 μ M.

Perhaps, further research will yield a method with which to maximize the drug's antibiotic effects and minimize the trauma it causes the auditory system. It is also conceivable that in the future, a method to promote hair cell regeneration could be developed. Some studies have shown that vitamin C and proper dietary intake may reduce the ototoxic effects of aminoglycosides on the hair cells (Lautermann et al., 1995). Other research has examined the effect of growth factors on hair cell proliferation (Saffer and Corwin, 1995; Yamashita et al., 1995). Hopefully, further research will provide the answers to the exact mechanism of action of aminoglycosides on hair cells and what makes hair cells susceptible to ototoxicity.

Conclusion

Well known for their ototoxic effects, the aminoglycosides destroy neural sensory hair cells. The injection of gentamicin into the otic capsules of *Rana pipiens* caused threshold shifts. In this study, hearing thresholds were measured using an ABR technique. This was the first study to selectively evoke a brainstem response from the two auditory organs using computer synthesized low and high frequency clicks. A broadband sinewave click was also used. No apparent differences in threshold between the AP, BP or the sinewave click were observed. Pre- and post-gentamicin injection thresholds were measured until thresholds recovered. After the intraotic administration of gentamicin, I found the dose- and potency-dependent thresholds returned to their pre-treatment thresholds within 14 days. The injection 200 μM gentamicin produced threshold shifts of 10 dB. The 300 μM injections resulted in 10-15 dB threshold shifts. Injections of 400 μM gentamicin yielded a threshold shift as great as 20 dB. Recovery of threshold began between

one and three days and total recovery occurred within 14 days post-injection for typical frogs.

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Appendix

Concentration Calculations

200 μ M Concentration:

$$([\text{con}]_{\text{initial}})(\text{Vol}_{\text{initial}}) = ([\text{con}]_{\text{final}})(\text{Vol}_{\text{final}})$$

$$([\text{con}]_{\text{initial}})(4\mu\text{l}) = (200\mu\text{M})(15\mu\text{l})$$

$$([\text{con}]_{\text{initial}}) = 750\mu\text{M}$$

300 μ M Concentration:

$$([\text{con}]_{\text{initial}})(\text{Vol}_{\text{initial}}) = ([\text{con}]_{\text{final}})(\text{Vol}_{\text{final}})$$

$$([\text{con}]_{\text{initial}})(4\mu\text{l}) = (300\mu\text{M})(15\mu\text{l})$$

$$([\text{con}]_{\text{initial}}) = 1125\mu\text{M}$$

400 μ M Concentration:

$$([\text{con}]_{\text{initial}})(\text{Vol}_{\text{initial}}) = ([\text{con}]_{\text{final}})(\text{Vol}_{\text{final}})$$

$$([\text{con}]_{\text{initial}})(4\mu\text{l}) = (400\mu\text{M})(15\mu\text{l})$$

$$([\text{con}]_{\text{initial}}) = 1500\mu\text{M}$$

Sample calculation for 400 μ M GS concentration:

1. We want $1500\mu\text{M} = 10^{-6}$ moles and $1500\mu\text{M} = .0015$ moles
2. We also want to make 10 ml of solution (0.010 L)
so, how many moles do we need to make a 0.0015 molar solution? (molar = mol/L)
3. $(.010 \text{ L}) \times (.0015 \text{ mol}/1.0 \text{ L}) = 0.000015$ moles GS
4. Now, how many grams in 0.000015 moles?
5. MW of GS = 464 grams/mole
6. $(0.000015 \text{ moles GS}) \times (464 \text{ g/mol GS}) = .00696 \text{ g} = 6.96 \text{ mg}$
7. However, the GS is only 652 μg gentamicin base per mg.
8. Thus, we divide 6.96 mg by .652 to yield 10.67 mg.
9. Thus, we add 10.67 mg of GS to 10 ml of HEPES-buffered saline solution.